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ON THE PROBLEM OF POSSIBLE OTHER FORMS OF CYTOCHROME P_{450} IN LIVER MICROSOMES

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SUMMARY

The absolute spectrum of cytochrome P_{450} is shown. The addition of a known substrate is shown to alter the absolute spectrum of this hemoprotein. The spectrum of P_{450} induced by polycyclic hydrocarbons appeared similar to that of the hemoprotein in the presence of substrate. This similarity is shown to be due to the binding of the inducer and/or its metabolites to the hemoprotein by the ability to displace them and restore the absolute spectrum to that of the native hemoprotein. This study indicates that P_{450} exists in only two forms, the native enzyme and the enzyme-substrate complex in prepared microsomes.

INTRODUCTION

Several studies on cytochrome P_{450} have been concerned with obtaining a greater understanding of the nature of this hemoprotein, with attempts ranging from studying the CO complex of the degradation product, P_{420} (refs. 1–3) and the stability of P_{450} (refs. 4, 5), to more recent attempts to obtain absolute spectra of P_{450} (refs. 6–9). This latter feat has previously been complicated by the presence of cytochrome b_5 in liver microsomes. The spectra of hepatic microsomal P_{450} obtained by the different laboratories are remarkably similar, despite the differences in procedures employed. In the study by KINOSHITA AND HORIE[6] and that by REMMER *et al.*[8], the interference by cytochrome b_5 was cancelled out by balancing microsomes of rats with higher P_{450} content (induced) against that of normal rats. In the study by NISHIBAYASHI *et al.*[7], and that by SCHENKMAN AND SATO[9], cytochrome b_5 was first removed from the liver microsomes. From these latter investigations it is clear that cytochrome P_{450} has an absorption peak at 420 mμ in the oxidized state.

These recent advances have paved the way for studies on the manner of substrate interaction with the hepatic microsomal mixed-function oxidase, the involvement of cytochrome P_{450} in this system[9,10], and the problem of whether there exists one P_{450} or two P_{450} types in liver microsomes[4,9–14,20,21].

The purpose of this communication is to describe studies aimed at answering the question of the number of P_{450} species existing in liver microsomes. It provides

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evidence that P_{450} exists in only two forms, a native enzyme form and a substrate-bound form, and that the two forms are interconvertible.

METHODS

Microsomes were prepared from the livers of rats pretreated with 3,4-benzpyrene (either 20 or 50 mg/kg body weight) daily for 4 – 5 days, or with 100 mg phenobarbital–Na per kg for 4 days, and were killed 24 or 48 h after the last injection. Rabbits were treated in a similar manner, using either 3,4-benzpyrene or 3-methylcholanthrene (12 mg/kg body weight) daily. Livers were chilled and perfused with 0.15 M NaCl to remove hemoglobin. Microsomes were prepared essentially as described before[15], but a 1:7 homogenate in 0.25 M sucrose–1 mM ethylenediamine tetracetate (EDTA) was used instead of the 1:10 homogenate to decrease volume requirements. The microsomal pellet obtained was washed once with 0.15 M KCl to remove all trace of hemoglobin. Absence of hemoglobin was ascertained by gassing half of a sample of microsomal suspension with carbon monoxide for 3 min and running a difference spectrum between the two; prior addition of small amounts of dithionite followed by aerobic shaking of the suspension also indicated an absence of methemoglobin in the suspension.

All chemicals were commercially obtained in the highest purity available and were not further purified.

Spectra were determined using a Leitz-Unicam SP-800 with an external recorder to increase sensitivity 20 fold, since spectral changes obtained on addition of substrates to hepatic microsomes is only about 10–15% of the absolute absorbance[9,10].

Benzpyrene or methylcholanthrene was extracted from the microsomes by diluting the suspension (about 10 mg protein/ml) with 3 vol. of 50% ethanol containing 0.25 M KOH (ref. 16), and extracting with 2.5 vol. of redistilled hexane by mechanical shaking for 30 min. After removal of the hexane phase, 0.5 vol. of 1 M HCl was added, followed by 2.5 vol. of hexane, and the sample was shaken for another 30 min to remove possible hydroxylated products of the polycyclic hydrocarbons. The hexane extracts of acid and alkaline microsomal suspensions were each concentrated and the content of polycyclic hydrocarbon estimated from absorption spectra, using extracts from normal animals as controls.

Spectra of the oxidized form of P_{450} in this communication were obtained by the method of KINOSHITA AND HORIE[6], as previously described[8], by balancing microsomal cytochrome b_5 and protein.

RESULTS

The absolute spectrum of cytochrome P_{450} is shown in Fig. 1A, balancing liver microsomes from phenobarbital-pretreated male rats against that from normal male rats. The resultant spectral band (solid curve) represents the absorption spectrum of 3.93 μM off-balance cytochrome P_{450} in the oxidized state, and has a maximum at 421 mμ.

The addition of hexobarbital to the sample cuvette caused a marked shift of the absorption maximum to 415 mμ, in agreement with the b_5-free preparation[9], as well as the appearance of an increase in absorption at 395 mμ which forms a new

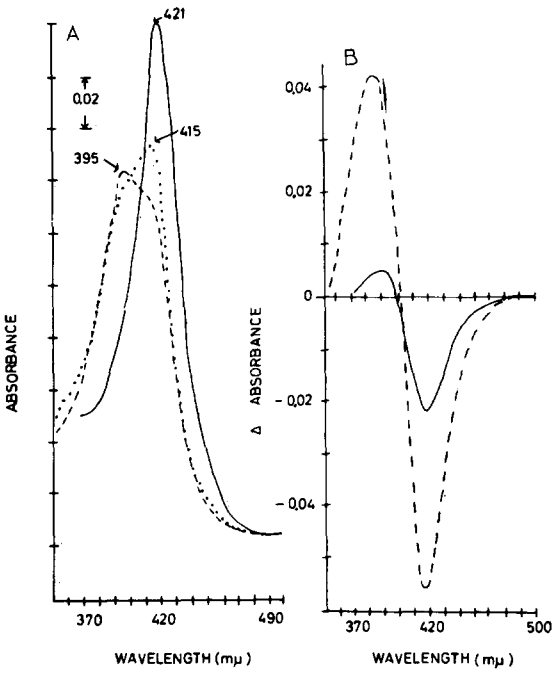

Fig. 1.A. Absolute spectra of phenobarbital-induced cytochrome P_{450}. Male rats were treated with 100 mg/kg per day for 4 days and killed 24 h after the last injection. The sample cuvette contained microsomes from phenobarbital-treated rats, 2.45 mg protein/ml, 5.7 μM P_{450} and 1.34 μM b_5, and the reference cuvette contained microsomes from normal male rats, 2.6 mg protein/ml, 1.77 μM P_{450} and 1.32 μM b_5, in 0.1 M Tris–HCl (pH 7.5) (———); 3.3 mM hexobarbital in the sample cuvette (— — —); 3.3 mM hexobarbital in both cuvettes (· · · ·). B. Difference spectra between normal rat liver microsomes containing 3.3 mM hexobarbital in the sample cuvette and the same preparation without hexobarbital (———) in the reference, and between microsomes from phenobarbital-pretreated rats with 3.3 mM hexobarbital and the same preparation without hexobarbital (— — —). The preparations in (A) were used. Benzpyrene hydroxylase activity of normal rats was 0.54 mμmole/min per mg microsomal protein, and of phenobarbital-treated rats 0.88. Hexobarbital oxidase activity was 3.8 mμmoles/min per mg protein for normal rats and 8.1 for phenobarbital-treated rats.

maximum (dashed curve). The addition of hexobarbital to the reference cuvette (dotted curve) causes an increase in absorption at 415 mμ with a slight decrease in absorption at 395 mμ. The decrease in the absorption at 421 mμ when hexobarbital was added to both cuvettes was about 20% of the overall absorption. The spectral change caused by the addition of hexobarbital is the type I spectral change[15], as seen when samples of microsomes containing hexobarbital are compared with the same samples without hexobarbital (Fig. 1B). When the magnitude of the 420 mμ trough was expressed on the basis of P_{450} concentration, a value of 0.0113 μM^{-1} P_{450} cm^{-1} was obtained for the microsomes from control rats, and 0.0101 for the microsomes of the phenobarbital-induced rats, both values being similar to that reported previously[9,15].

Other compounds are known to elevate the content of P_{450} in liver microsomes[17]. Fig. 2 shows the effect of pretreatment of male rats with the polycyclic hydrocarbon

inducer 3,4-benzpyrene (a single injection of 50 mg/kg) 24 h before killing the rats. In Fig. 2A, as with phenobarbital pretreatment, the benzpyrene-induced P_{450} has a peak in the oxidized form at 421 mμ. However, the absorption spectrum (solid line) also has a prominent shoulder at 394 mμ, as in Fig. 1A after substrate (hexobarbital) addition. The addition of hexobarbital to the sample cuvette causes an increase in the absorption peak at 394 mμ, and a simultaneous decrease in the magnitude of absorption at 421 mμ. The addition of hexobarbital to the reference cuvette (normal rat microsomes) returned the absorption maximum to 421 mμ, but to a slightly greater magnitude than before (dotted curve), and removed most of the shoulder at 394 mμ.

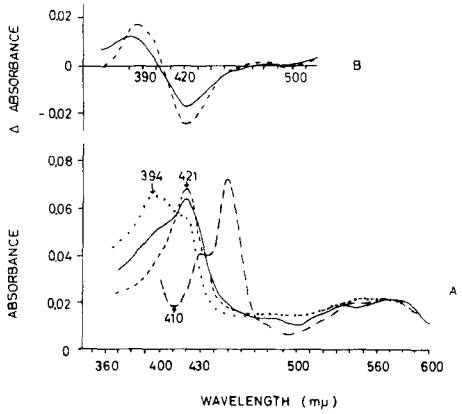

Fig. 2.A. Absolute spectra of 3,4-benzpyrene-induced cytochrome P_{450}. Male rats were injected with 50 mg of benzpyrene per kg body weight 24 h before being killed. The sample cuvette contained microsomes from benzpyrene treated rats, 2.98 mg protein per ml. 2.4 μM P_{450} and 1.32 μM b_5, and the reference cuvette contained microsomes from normal rats, 2.6 mg protein per ml, 1.77 μM P_{450} and 1.32 μM b_5, in 0.1 M Tris–HCl (pH 7.5) (———); 3.3 mM hexobarbital in sample cuvette (· · · · ·); 3.3 mM hexobarbital in both cuvettes (— — —); CO and $Na_2S_2O_4$ in each cuvette (— —). B. Difference spectra between microsomes from benzpyrene-induced rats with 3.3 mM hexobarbital and the same preparation without hexobarbital (———), and between normal rat liver microsomes with 3.3 mM hexobarbital and the same preparation without hexobarbital. The preparations shown in (A) were used. Benzpyrene hydroxylase activity in the pretreated animal liver microsomes was 1.98 mμmoles/min per mg protein, and hexobarbital oxidase activity was 3.2 mμmoles/min per mg microsomal protein. Activity of normal rat liver microsomes was that shown in Fig. 1A.

The effect of hexobarbital on each individual preparation is shown in Fig. 2B, and indicates that the magnitude of the type I spectral change is lower in the microsomes of benzpyrene-treated animals (0.007 μM^{-1} $P_{450} \cdot cm^{-1}$) than in the normal animals (0.0115).

The effect of pretreatment of adult male rats with benzpyrene is somewhat different from the effect of pretreatment of female rats with this compound. Fig. 3A shows the absorption of the off-balance P_{450} induced in the female (solid curve). As in Fig. 2A, there is a distinct peak in the absorption spectrum at 394 mμ. However, addition of hexobarbital to the sample cuvette (dashed curve) decreases the height of the 394 mμ peak with a subsequent increase in absorption at 421 mμ in a reverse fashion to that seen in Fig. 2A. The addition of more hexobarbital to the sample

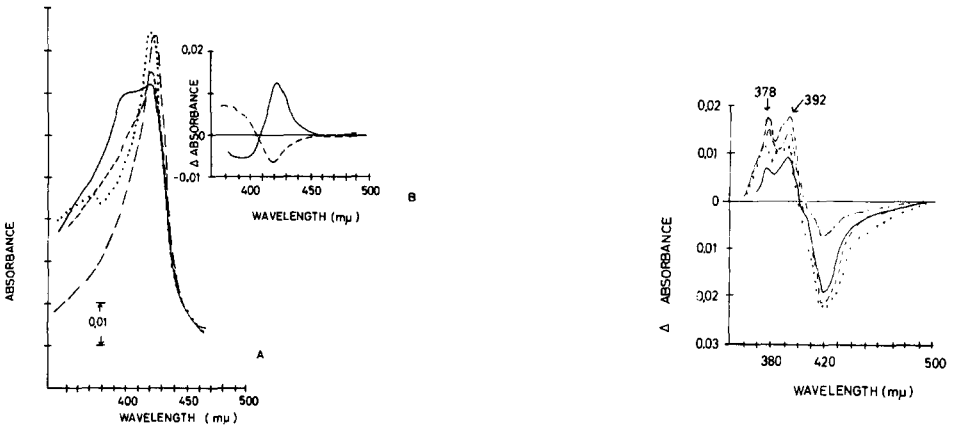

Fig. 3.A. Absolute spectra of 3,4-benzpyrene-induced cytochrome P_{450} in female rat liver microsomes. Female rats were pretreated for 5 days with daily injections of 50 mg benzpyrene, and were killed on the 6th day. The sample cuvette contained microsomes from the treated animals, in 0.1 M Tris–HCl (pH 7.5), 2.74 mg protein/ml, 2.42 μM P_{450} and 1.1 μM b_5, and the reference cuvette contained microsomes from normal rats, 2.5 mg protein/ml, 0.88 μM P_{450}, and 1.09 μM b_5 (———); 7 mM hexobarbital in sample cuvette (– – –); 14 mM hexobarbital in sample cuvette (· · · ·); 14 mM hexobarbital in sample cuvette and 7 mM hexobarbital in the reference cuvette (– – – –). B. Difference spectra between microsomes from normal female rats with 7 mM hexobarbital and the same preparation without hexobarbital (— — —), and between microsomes from benzpyrene-treated animals with 14 mM hexobarbital and the same preparation without hexobarbital. The same preparations shown in (A) were used.

Fig. 4. The formation of the type I spectral change by addition of 3,4-benzpyrene to rat liver microsomes. Microsomes were used from male rats pretreated for 4 days with 20 mg/kg of benzpyrene per day, followed by an injection of 50 mg benzpyrene per kg on the 5th day. The rats were killed 24 h after the last injection, and liver microsomes were suspended in 0.1 M Tris–HCl (pH 7.5), to 2.32 mg protein per ml, 1.26 μM b_5 and 2.3 μM P_{450}. 5 μl of excess benzpyrene in ethanol was added to the sample cuvette, and ethanol to the reference cuvette (volume 3 ml/per cuvette) (———); 5 μl more benzpyrene and ethanol was added to sample and reference cuvettes (– – – –); 3.3 mM hexobarbital was added to sample cuvette containing benzpyrene, (· · · ·); 3.3 mM hexobarbital was added to the reference cuvette (—·—).

cuvette (dotted line) increases the magnitude of the 421 mμ peak further, as does addition of hexobarbital to the reference cuvette. In Fig. 3B, the difference spectrum of hexobarbital effects in each preparation indicates the reason for the observed effects. Although normal female rat liver microsomes show the type I spectral change (0.0068 μM^{-1} $P_{450} \cdot cm^{-1}$), the microsomes from benzpyrene pretreated female rats show the modified type II spectral change[15], which is the reverse of the type I spectral change and by this in the absolute spectrum the shoulder at 394 mμ decreases and the absorption at 421 mμ increases (Fig. 3A). This is opposit to experiments with male rats (Fig. 2) where addition of hexobarbital to microsomes of untreated and benzpyrene treated rats in the difference spectrum resulted both in type I spectral changes (Fig. 2B).

Fig. 4 shows the effect of the addition of benzpyrene in ethanol to liver microsomes; ethanol was added to the microsomal preparation in the reference cuvette as a solvent control. The resultant difference spectrum shows a type I spectral change, indicating that the polycyclic hydrocarbon is bound in the manner of other known substrates[15], possibly even to a greater extent.

HILDEBRANDT, REMMER AND ESTABROOK[11] have reported the presence of a new

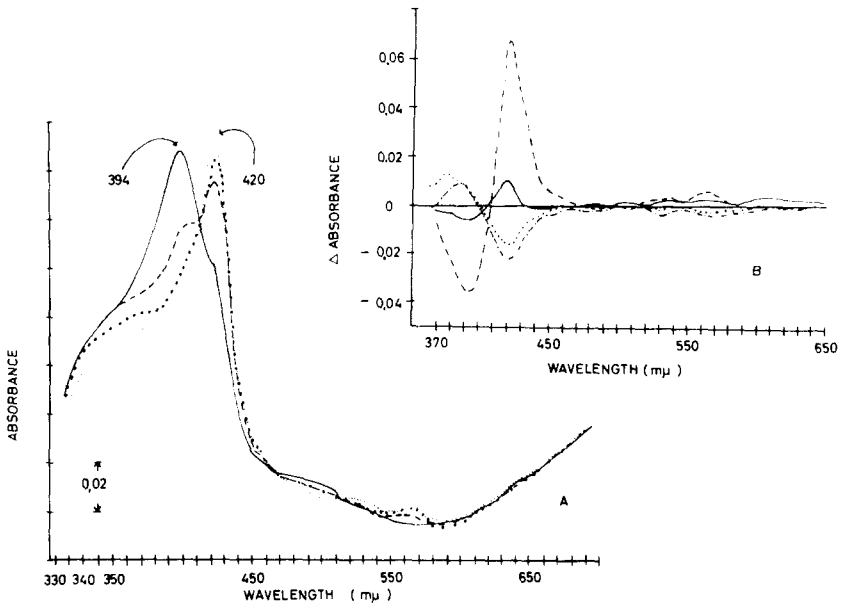

Fig. 5. A. Absolute spectra of 3-methylcholanthrene-induced cytochrome P'_{450} in male rabbit liver microsomes. Rabbits were injected with 12 mg methylcholanthrene per kg body weight at 12-h intervals and killed 40 h after the fourth injection. The sample cuvette contained microsomes from the treated animal suspended in 0.1 M Tris–HCl (pH 7.5), 5.3 mg protein/ml, 2.73 μM P_{450} and 1.24 μM b_5 and the reference cuvette contained microsomes from normal rabbit, 7.1 mg protein/ml, 1.32 μM P_{450} and 1.24 μM b_5 (———); 10 mM hexobarbital in the sample cuvette (– – – –); 15 mM hexobarbital in the sample cuvette (· · · ·); 15 mM hexobarbital in the sample cuvette and 6 mM hexobarbital in the reference cuvette (fine dots). B. Difference spectra between microsomes of methylcholanthrene-treated rabbit with 3.3 mM hexobarbital (— — —) or 15 mM hexobarbital (– – – –) and the same preparation without hexobarbital and between microsomes of normal rabbit containing 3.3 mM hexobarbital (— · — ·) or 6.6 mM hexobarbital (· · · · ·) and the same preparation without hexobarbital.

form of P_{450} observed after pretreatment of rabbits with 3-methylcholanthrene. Upon repeating these experiments, it was found that the 394 peak obtained is not a new form or type of P_{450} at all. Fig. 5A shows the spectrum of the off-balanced (induced) P_{450} after pretreatment with 3-methylcholanthrene (solid curve). In addition to the very high maximum present at 394 mμ, there is a distinct shoulder at 420 mμ. The addition of 10 mM hexobarbital to the microsomal suspension in the sample cuvette (induced) shifted the 394 mμ maximum to 420 mμ (dashed curve) as in Fig. 3A, and also caused the appearance of the two visible bands of the hemoprotein at 530 and 570 mμ. There did not appear to be alterations in absorption in the region of 650 mμ. The addition of more hexobarbital to the sample cuvette further increased the magnitude of the 420 mμ peak and simultaneously further decreased the magnitude of the 394 mμ absorption (dotted curve), as did addition of hexobarbital to the reference cuvette. The effect of hexobarbital on each preparation alone is shown in Fig. 5B. Note that while addition of hexobarbital to the microsomes from the control male rabbit caused the type I spectral change to appear, the same amount of hexobarbital to liver microsomes of methylcholanthrene-treated rabbits produced a small modified type II spectral change, which could be increased markedly by increasing

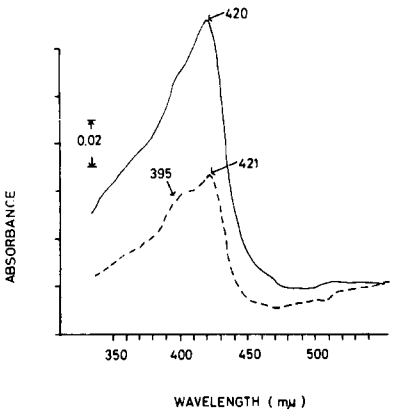

Fig. 6. Absolute spectra of 3,4-benzpyrene-induced P_{450} in microsomes of female rats treated differently. ———, The sample cuvette contained microsomes from rats treated for 4 days with 50 mg/kg benzpyrene daily, with animals being killed 48 h after the last injection; protein in suspensions was 2.58 mg/ml, 1.09 μM b_5 and 2.58 μM P_{450}, and the reference cuvette contained microsomes of normal rats, 2.5 mg protein/ml, 1.09 μM b_5, and 0.88 μM P_{450}. – – – –, The sample cuvette contained liver microsomes from female rats daily treated for 5 days with 50 mg/kg injections of benzpyrene and killed 25 h after the last injection; 2.74 mg protein/ml, 1.1 μM b_5 and 2.42 μM P_{450}. The reference cuvette contained the same suspension as in the other curve.

the concentration of hexobarbital; more hexobarbital added to the control microsomes only slightly decreased the type I spectral change.

Although it is possible to view off-balanced P_{450} with the KINOSHITA AND HORIE procedure[6], the use of this technique does not allow the estimation of the absolute extinction of this hemoprotein. Fig. 6 shows this deficiency in the technique quite clearly. The two different preparations of microsomes used here are from benzpyrene-pretreated rats, but they are recompared against the same preparation of normal rat liver microsomes. Although the microsomes from 5-days pretreated rats had 10% less off-balance P_{450} than the 4-days pretreated rats, the absorption of its off-balanced P_{450} was 50% lower at 420 mμ.

When microsomes in alkali were extracted with organic solvents to remove any of the polycyclic hydrocarbon that might be present, the unmistakable absorption spectrum of these compounds was seen, even when the animals were killed 40 h after the last injection. The amount of benzpyrene-like absorption removed from microsomes of rabbits, pretreated with 10 mg benzpyrene per kg for 5 days and killed 40 h after the last injection, was equivalent to about 2 mμmoles per 100 mμmoles of P_{450}. About 4 mμmoles of methylcholanthrene-like absorption per 100 mμmoles P_{450} was extracted from the microsomes used in Fig. 5. Subsequently acidified microsomal suspensions did not yield detectable amounts of polycyclic hydrocarbon-like absorption.

DISCUSSION

Previously[9,10], we suggested that cytochrome P_{450} exists in liver microsomes in two forms. One form, the "420 form", was suggested as being the native hemoprotein in the mixed-function oxidase system, and having an absorption maximum at 419–

420 mμ. The other form, the "390 form", having an absorption peak at 394 mμ, represents the enzyme–substrate complex. The relationship between these two forms is seen by the type I spectral change[15], where the 420 mμ absorption was seen to disappear with the simultaneous appearance of an absorption band at 390 mμ, in difference spectrum.

The two forms were shown to be interconvertible[15] when substrates were removed from the microsomes. However, in the event that substrates are extremely water-insoluble, it would not be possible to wash them free of the microsomes. In the absolute spectrum of P_{450} (Fig. 1A) it was shown that substrates cause the appearance of a shoulder at 394 mμ. If substrates are already bound to the enzyme, the absolute spectrum of off-balance P_{450} would be expected to show a shoulder or peak in this region. Thus, in Figs. 2, 3 and 5, the off-balance P_{450} induced by pretreatment with polycyclic hydrocarbons, exhibits the 394 mμ band, in different relative magnitudes, due probably to different amounts of polycyclic hydrocarbon, endogenous substrates, and hydroxylated metabolites of these compounds bound to the enzyme. That the polycyclic hydrocarbons do interact as normal substrates was shown in Fig. 4; it has already been reported that steroids, which may be endogenous substrates, bind to the mixed-function oxidase[15].

The addition of hexobarbital to liver microsomes normally causes a type I spectral change, with the magnitude of the trough being about 0.011 absorbance units per μM heme per cm (refs. 9, 10, 15). In benzpyrene-treated male rat liver microsomes, this value is reduced somewhat (about 35%). The effect is due either to benzpyrene or its metabolites already bound to some of the enzyme. In previous studies (J. B. SCHENKMAN, H. REMMER AND R. W. ESTABROOK, unpublished observations) it was also observed that the K_m for the substrate aminopyrene, the K_s (spectral dissociation constant) for aminopyrene, and the K_s for hexobarbital were all increased markedly in rats pretreated with benzpyrene.

Thus it appears that at least some of the differences in liver microsomes from animals pretreated with polycyclic hydrocarbons can be attributed to either the presence of these compounds or their metabolites. It is also possible that the presence of the very large polycyclic hydrocarbon molecule is the cause of the displacement of the absorption peak of the 450 mμ CO complex, as reported by ALVARES *et al.*[14], to 448 mμ.

In the case of the female normal rats, the decreased spectral change has already been suggested as being due to the presence of some endogenous substrate which acts as an inhibitor increasing the K_m and K_s for added substrates, or to the presence of a different binding protein[18].

The fact that hexobarbital, in concentrations 5 or 6 fold higher than necessary to cause type I spectral change, can cause the conversion of the 390 form of P_{450} back to the 420 form in benzpyrene-treated female rats and in methylcholanthrene-treated male rabbit liver microsomes, tends to strengthen the suggestion that in these cases, the 394 mμ band is the result of a binding of the inducer or its metabolite to the enzyme. Although only a small amount of polycyclic hydrocarbon could be extracted from the microsomes after addition of alkali, and no hydrocarbon-like absorption could be extracted after acidification, the hydroxylated polycyclic hydrocarbons are reportedly very tightly bound and difficult to remove[16]. The necessity for high levels of hexobarbital to cause conversion to the 420 form may reflect a difference in affinity

between hexobarbital and whatever is already bound to the enzyme.

Recently, NISHIBAYASHI *et al.*[7] have listed the extinction coefficient of P_{450} in the oxidized form as about 108 $mM^{-1} \cdot cm^{-1}$. The value calculated from the data of SCHENKMAN AND SATO[9] comes to about 150 $mM^{-1} \cdot cm^{-1}$, using a similar preparation. The problem of determining an extinction coefficient with such an impure preparation as microsomes embraces the obvious problem of balancing the turbidity, as well as the less obvious problem of not knowing the amount of P_{450} present in the 390 form. It is possible that the true extinction coefficient of this mammalian hemoprotein will not be known until its solubilization and purification from the membranes.

This binding of inducer compounds to the enzyme would also explain the decrease in oxidation rate of substrates of the enzyme system[19]; the level of inhibition varying with the amount of inducer, or its metabolites, bound to the enzyme.

REFERENCES

1 T. OMURA AND R. SATO, *J. Biol. Chem.*, 239 (1964) 2379.
2 H. S. MASON, J. NORTH AND M. VANNESTE, *Federation Proc.*, 24 (1965) 1172.
3 Y. ICHIKAWA AND T. YAMANO, *Biochim. Biophys. Acta*, 131 (1967) 490.
4 Y. IMAI AND R. SATO, *Europ. J. Biochem.*, 1 (1967) 419.
5 Y. ICHIKAWA, T. UEMURA AND T. YAMANO, in *Symposium on Cytochromes, Osaka, 1967*, Tokyo University Press, 1967, p. 334.
6 T. KINOSHITA AND S. HORIE, *J. Biochem.*, 61 (1967) 26.
7 H. NISHIBAYASHI, T. OMURA, R. SATO AND R. W. ESTABROOK, in *Symposium on Cytochromes, Osaka, 1967*, Tokyo University Press, 1967, p. 351.
8 H. REMMER, R. W. ESTABROOK, J. SCHENKMAN AND H. GREIM, *Arch. Pharmakol. Exptl. Pathol.*, 259 (1968) 98.
9 J. B. SCHENKMAN AND R. SATO, *Mol. Pharmacol.*, in the press.
10 J. B. SCHENKMAN, *Z. Physiol. Chem.*, 349 (1968) 36.
11 A. HILDEBRANDT, H. REMMER AND R. W. ESTABROOK, *Biochem. Biophys. Res. Commun.*, 30 (1968) 607.
12 Y. IMAI AND R. SATO, *Biochem. Biophys. Res. Commun.*, 23 (1966) 5.
13 N. E. SLADEK AND G. J. MANNERING, *Biochem. Biophys. Res. Commun.*, 24 (1968) 668.
14 A. P. ALVARES, G. SCHILLING, W. LEVIN AND R. KUNZMAN, *Biochem. Biophys. Res. Commun.*, 29 (1968) 521.
15 J. B. SCHENKMAN, H. REMMER AND R. W. ESTABROOK, *Mol. Pharmacol.*, 3 (1967) 113.
16 D. A. SILVERMAN AND P. TALALAY, *Mol. Pharmacol.*, 3 (1967) 90.
17 H. REMMER, H. GREIM, J. B. SCHENKMAN AND R. W. ESTABROOK, *Methods Enzymol.*, 10 (1967) 703.
18 J. B. SCHENKMAN, I. FREY, H. REMMER AND R. W. ESTABROOK, *Mol. Pharmacol.*, 3 (1967) 516.
19 A. H. CONNEY, C. DAVISON, R. GASTEL AND J. J. BURNS, *J. Pharmacol. Exptl. Therap.*, 130 (1960) 1.
20 H. REMMER, H. GREIM, A. HILDEBRANDT, *Arch. Pharmakol. Exptl. Pathol.*, 260 (1968) 189.
21 H. REMMER, *Z. Physiol. Chem.*, 349 (1968) 33.